CHITOSAN, STARCH AND PECTIN FOR MINERAL-POLYMERIC MATRICES

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Abstract

Biomaterials for regenerative medicine may include mineral-polymer matrices based on the starch, chitosan and CP with different Ca/P ratios. Crystals connecting with the polymer matrix are combined into 200-300 μm agglomerates, which are evenly distributed over the film surface. This paper successfully has demonstrated that the films of starch-chitosan mixture

don't have a toxic effect. Research results show that pectin hydrogels can be used as biomaterials for cell delivery in regenerative medicine, etc. Modified pectins and nanopectins can be obtained from agricultural raw materials or from industrial pectin. The method of pectin modification is analyzed by HPLC, SEC, etc. In most cases, modified pectin has a nano rodlike structure. According to our results samples of chitosan, pectin, and modified pectin are demonstrated a significant induction of cell differentiation during experiments. As shown, cells treated with adipogenic agents which also contained 1000 μg / ml of the

modified pectin accumulated more 20 ± 2%
of intracellular triglycerides compared to the control sample treated with the same agents.

Introduction

With increasing demand for materials for medicine, the field of tissue engineering has become very popular all over the world. But the development of biomaterials for regenerative medicine is still considered a new field [1]. The creation of materials for medicine is of the primary task for researchers in the field of chemistry and medicine. Hydrogels have been extensively studied as synthetic extracellular matrices (ECMs) to use in tissue engineering and regenerative medicine mainly due to their high viscoelastic and diffusive transport properties. These properties make them similar to the ECM of many tissues [2]. Many biomaterials have been explored. Biocompatible materials involving biopolymers, such as collagen and poly-lactic acid (PLA), are the most widely studied materials for the regeneration of damaged tissues, acting as artificial supports for cell growth [3]. Biopolymers, such as polysaccharides, have been used to create materials for the field of tissue engineering too. Numerous studies have demonstrated that polysaccharides have similar properties with the extracellular matrix and the property of cytocompatibility. Similarly, chitosan is widely investigated to obtain hydrogels for tissue engineering. The chitosan and its derivatives are promising for the creation of implants
and carriers of medicines such as gels, films, fibers, sponges and other forms of matrix, due to its ability to form films, fibers and due to its unique sorption and complexing properties [4]. It may be useful in medicine, for example, in bandages to reduce bleeding and as an antibacterial agent; it can also be used to help deliver drugs through the skin. Based on recent works, researchers are actively studying porous biodegradable matrices for their application in the tissue engineering. The use of natural/bio fiber reinforced composites has rapidly expanded due to the availability of such renewable resources, for use as reinforcing composites [5, 6]. The Chitosan has significant disadvantages, for example, a relatively low rate of resorption in the body, as well as a poor elasticity of sponges and films, so it is difficult to use in the medicine. It is recognized that one of the methods to eliminate these disadvantages is to use (chitosan blends) [4] a mixture of chitosan having more resorbed macromolecules. Some of studies have shown that calcium phosphate (CP) contributes

to the healing of wounds.

The aim of our investigations was the study of mineral-polymer matrices based on the starch, chitosan and CP with different Ca/P ratios.

Some authors consider that the fast degradation rate is a relative property, as it is dependent on the aim of the study; it could be interesting for an application in which fast cell proliferation and production of the extracellular matrix are desirable. The results of the work show how versatile pectin hydrogels can be, and also how fast they can degrade without the need for extensive chemical modifications [7]. Considering the described benefits, it was proposed to use the pectin and modified pectin, as reinforcing composites with other biodegradable polymer matrices. Modified Pectin, modified citrus pectin, also known as MCP, has a reduced molecular weight compared to regular citrus pectin, a mostly linear homogalacturonan chain, which are easily processed by the digestive system and absorbed into the bloodstream. Scientists believe that MCP is working by inhibiting two key processes involved in cancer progression: angiogenesis and metastasis. [8,9] Modified pectin's and nano-pectins being vary from the semi-crystalline to highly crystalline material and differ from the initial pectin by GE, MW [10]. The classification and properties of nanostructured materials and systems [11] essentially depend on the number of dimensions which lie within the1 nanometer range. The process ability of MCP, Nano-pectin is similar to conventional pectin-based polymers. Preliminary data suggests that modified pectin and nano-pectin are beneficial for nanostructured materials and systems and mineral-polymer matrices.

The other aim of our investigations was the study of structure changes by the production of modified pectins.

Material and methods

Materials

Cell Analysis Kits (SYTO 9), propidium iodide (Analytical grade, Sigma-Aldrich) were used. 3T3-L1 adipocytes was obtained from the American Type Culture Collection (Manassas, VA, EE.UU.). Dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), insulin, cristal violet were obtained from Sigma-Aldrich. The culture medium (DMEM) was prepared in laboratories of GmbH, Linz, (Austria). Sodium pyruvate and EDTA-trypsin were obtained from the Invitrogen company (Carlsbad, CA). AdipoRed™ was obtained from Lonza (Walkersville, MD, EE.UU.). Samples of the polygalacturonic acid, pectin (Sigma Aldrich, EEUU), chitosan extract

(Monteoleder SL, Elche), modified pectin were used. Samples of the modified pectin (PM) made by the company Extractos de Citricos SL and Mitra Solutions SL (supplied by G. Ignatieva). The purified hydroxychalcone from cinnamon (MHCP) was prepared as described by Solonenko AP and Golovanova OA [12]. Bovine serum albumin was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). 2-deoxy-D[1,2-^{3}H] glucose, D[^{14}C] glucose, uridine diphosphate D[^{14}C] glucose and [^{32}P] adenosine-5-triphosphate were obtained from ICN Radiochemicals (Costa Mesa, CA). Antibodies against the insulin receptor ß subunit and GSK-3ß were purchased from Upstate Biotechnology (Lake Placid, NY). Dulbecco's modified Eagle's medium (DMEM), donor calf serum, sodium pyruvate, glutamine, trypsin-ethylene diamine tetraacetate, penicillin, streptomycin and Leibovitz L-15 medium were purchased from Life Technologies (Grand Island, NY). Fetal bovine serum was purchased either from Life Technologies or Novagen (Madison, WI). Wortmannin and LY294002 were purchased from Calbiochem (La Jolla, CA). Protein A/G plus Sepharose was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Other chemical and buffer components were purchased from either Sigma or Fisher Scientific Co. (Pittsburgh, PA).

Methods

The investigation of cytotoxicity materials was carried out by the direct contact. [13] The dental pulp stem (DPS) cells were used for the study at 4-6 stages. The method of fluorescent staining of SYTO 9 cells and propidium iodide was used to determine the viability of cells. The mixture of SYTO 9 green-fluorescent nucleic acid stain and propidium iodide, which is a popular red-fluorescent nuclear and chromosome counterstain, was used for viability determinations. This mixture was used as a calibrated suspension of microspheres for accurate measurements

The cytotoxic characteristic of material extracts was study in triplicate using the MTT test. The MTT assay is a colorimetric assay for assessing cell metabolic activity. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The induction of differentiation was by standard protocols (14). The cytotoxicity of the samples was determined by the crystal violet method as described (Ishiyama et al., 1996) (15) with plates (SPECTRO star Omega, BMG LABTECH). Statistical analyses were performed using Graph Pad Prism 5.0 software (version 5.0a). All tests were performed using a 95% confidence interval and statistically significant differences are marked with ($p < 0.05$), ($p < 0.005$) or ($p < 0.001$).

Results and discussion

The phase composition of the films depends on the ratio of Ca / P and of pH of the reaction medium. It was found that needle crystals corresponding to the brushite phase (12)

(dicalcium phosphate dihydrate, DCPD) are formed in the polymer solution at pH 5.5 and at a ratio of 1:1 (Ca / P). Crystals, that connecting with the polymer matrix, are combined into agglomerates of 200-300 μm, which are evenly distributed over the surface of the film. Earlier it was studied the adsorption of some amino acids (Ser, Glu, Gly, Asp, Ala, Arg, Tyr) included in the oral fluid on calcium hydrogen phosphate dihydrate (similar to the mineral brushite Ca_2HPO_4 x $2H_2O$). It is found that in all cases the interaction is described in terms of a monomolecular Langmuir adsorption (r^2 = 0,97-0,99) (16). The apatite phase (AP) is formed when pH of the medium and the ratio of Ca/P are increased to 7 and 1.5, respectively (Fig. 1). In this case, crystals are characterized by irregular shapes, the size of up to 2 μm and chaotic locations in the polymer matrix.

The treatment of films of starch-chitosan mixtures (1: 2) with an aqueous solution of ammonia results in partially cross-linked forms. Crosslinked films are characterized by several parameters, such as breaking elongation, strength characteristics. These obtained films are showed higher strength characteristics than the breaking elongation. The breaking elongation is decreased by 2 times. At the same time the elastic modulus and the rupture stress are increased approximately by 2 times.

Another part of investigations studied the pharmaceutical and anti-cancer properties of pectin (modified pectin, unmodified pectin) and chitosan. Recent research results show that pectin hydrogels can be used as biomaterials for cell delivery in regenerative medicine, etc. Generally, MCP has a molecular weight of 15400 g/mol. MCP is mainly a linear homogalacturonan with 3.8% degree of esterification and about 10% of the rhamnogalacturonan II. Modified pectin and Nano-pectin can be obtained from raw agricultural materials [17] or from industrial pectin (table 1) by acid, alkali [10] or enzyme treatments.

The pectin modify procedure from industry pectin was analyzed by HPLC (Figure 2, 3, 4, 5). The dates show characteristic peaks around 5,6min; 8,1min; 8,4min; 8,9min. The area of these peaks is changed. At the first stage impurities, non-pectin compounds and branched sections are removed (Figure 2, A1, B1, A2). At the second stage sugar compounds and amorphous regions are removed (Figure 2C1). Then the fine structure and sugar regions are modified (Figure 2, D1, D2). The chromatograms E1 (DAD), E2 (RID) of figure 2 show the process of pectin chain destruction. The chromatograms G1, K1 (DAD), G2, K2 (RID) of figure 3 show the modify process of hydrophobic and hydrophilic properties of polymer chains. The peak at 5,658min (Fig. 4C, 5C) shows the small MCP polydispersity index. The peaks at 8,052 and 8,144min (Fig. 4A, 5A) show the semi-crystalline / crystalline nature of these MCPs. The HPLC of MCP obtained by alcohol precipitation and spray drying is shown in Figure 4 and 5, respectively.

Figure 4C, 4A, 4B shows the peaks at t_r=5,628min, at t_r=8,906min, at t_r=8,351min, t_r=11,902min. Figure 5C, 5A, 5B shows the peaks at t_r=5,628min, at t_r=8,906min, at t_r=8,106min, t_r=11,902min, t_r=18,449min. By optimization of the extraction, drying temperatures and other treatments MCP is obtained with a molecular weight of 10-20 KDa, a degree of polymerization of 30-70 units and a degree of esterification of less than 50% (Table. 2). The most common compounds of MCP I, MCP II (Tab. 2) are – pectin, oligo-, homogalacturonan acid, unbound saccharides. The precipitation method reduces by 30% the content of pectin, of oligogalacturonic acid, of unbound saccharides and by 52% the content of minor unbound galacturonic acid (MCP I) when compared to MCP II (Fig. 3). These pectin's were characterized by SEC (size exclusion chromatography). This analysis of the MPCI, MCPII were done to determine its structure as well as to obtain a better understanding of the materials' crystallinity.

Thus, a mixture of nano- and microcrystalline pectin is obtained, where the particles of microcrystalline oligo-galacturonic acid are predominated. It can be seen that MPC appeared in the nanorodlike structure. The diameter and length of microcrystals are in the range of 5-6 μm and 100-200 nm, respectively. It is also possible to say that the theoretical dimensions are equivalent to obtained dates.

Table 1. Dates of industrial pectins.

No.	Molecular weight, KDa	Grade of esterification, %
1	40,4	61,5
2	41,2	81,5
3	28,8	33,0
4	51,8	74,5
5	61,7	67,0
6	53,0	60,0
7	53,4	74,5
8	22,1	47,5
9	53,1	40,0
10	30,6	11,0

Table 2. Analysis of the dried modified pectin.

Drying	pH	MW, KDa	Gal A,%	GE,%	Pectin, %	Content,%					
						Na	K	Glu	Fruc	Saccharides	Acid organic
Alcoholic precipitation	3’0	12	--	--	90,5	0	0	0	0	0	0
Spray dryer	3’0	11	61’1	50’8	69,7 ±1,0	0	0	1,8± 0,1	0	19,0±0,3	1,70 ±0,01

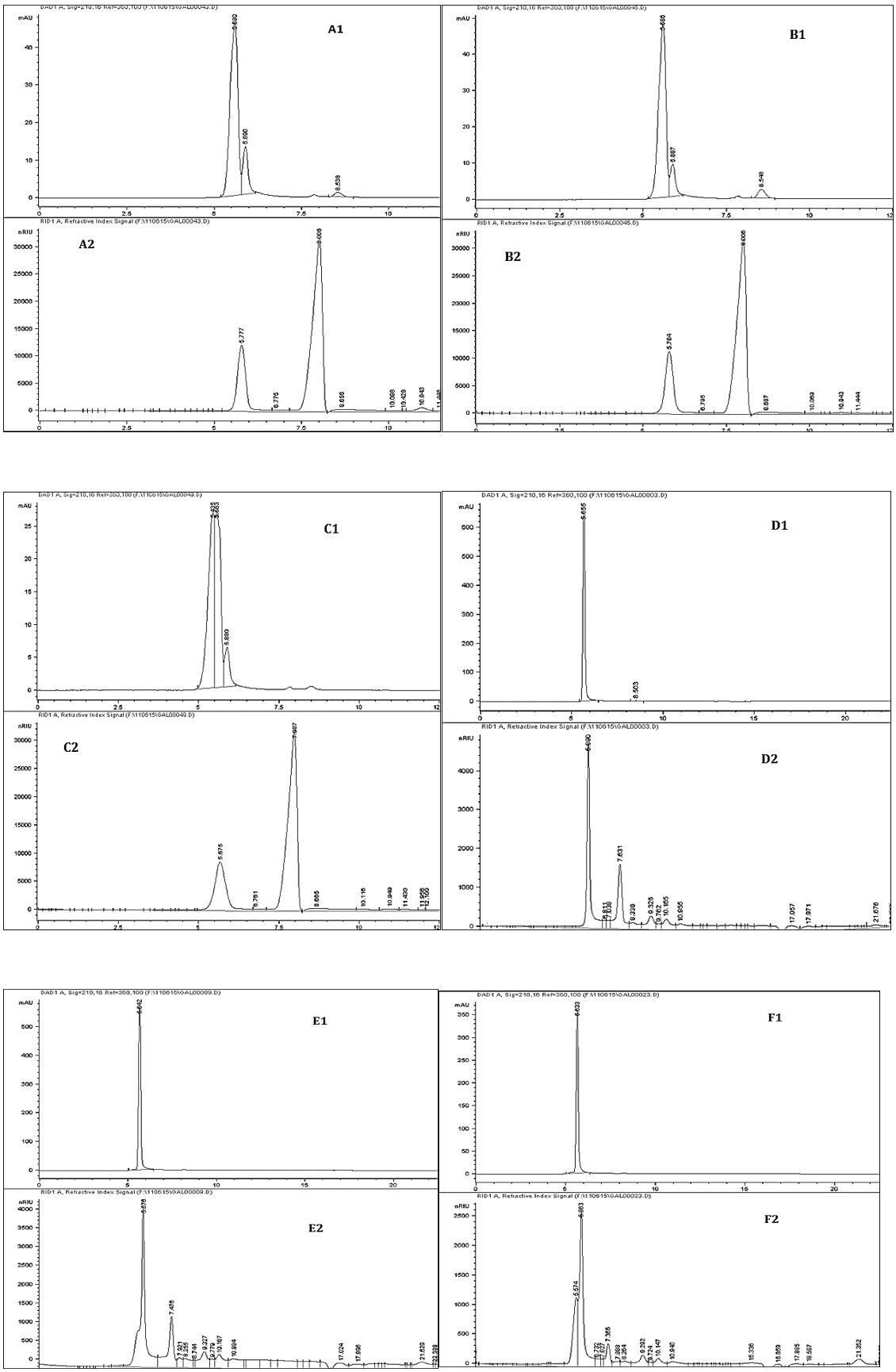

Figure 1. Pectin peaks are characteristic. Pectin samples were obtained by alcohol precipitation. Study of the characteristic peaks of various MCPs that show a low polydispersity index and a semi-crystalline / crystalline nature of this reinforcement.

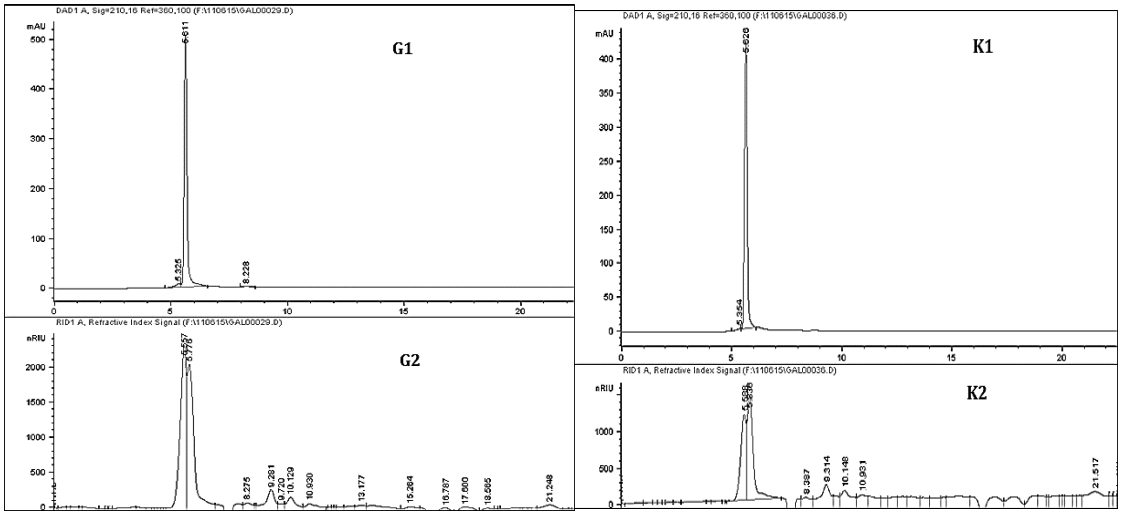

Figure 2. Pectin peaks are characteristic. Pectin samples were obtained by spray drying. Study of the characteristic peaks of various MCPs that show a low polydispersity index and a semi-crystalline / crystalline nature of this reinforcement.

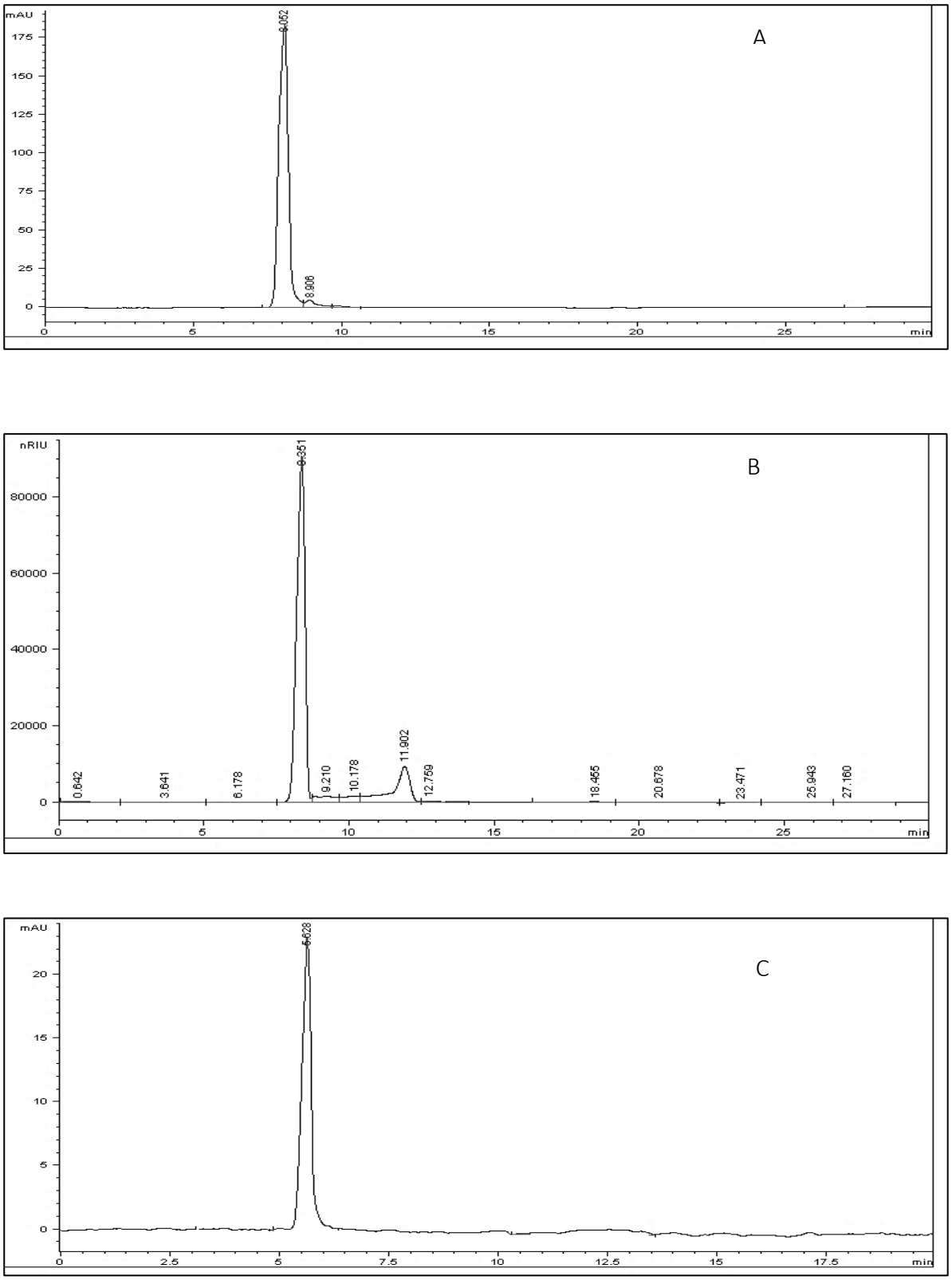

Figure 3. HPLC chromatogram of organic acids (A), sugars (B) and pectin (C) of the modified pectin are obtained by alcohol precipitation.

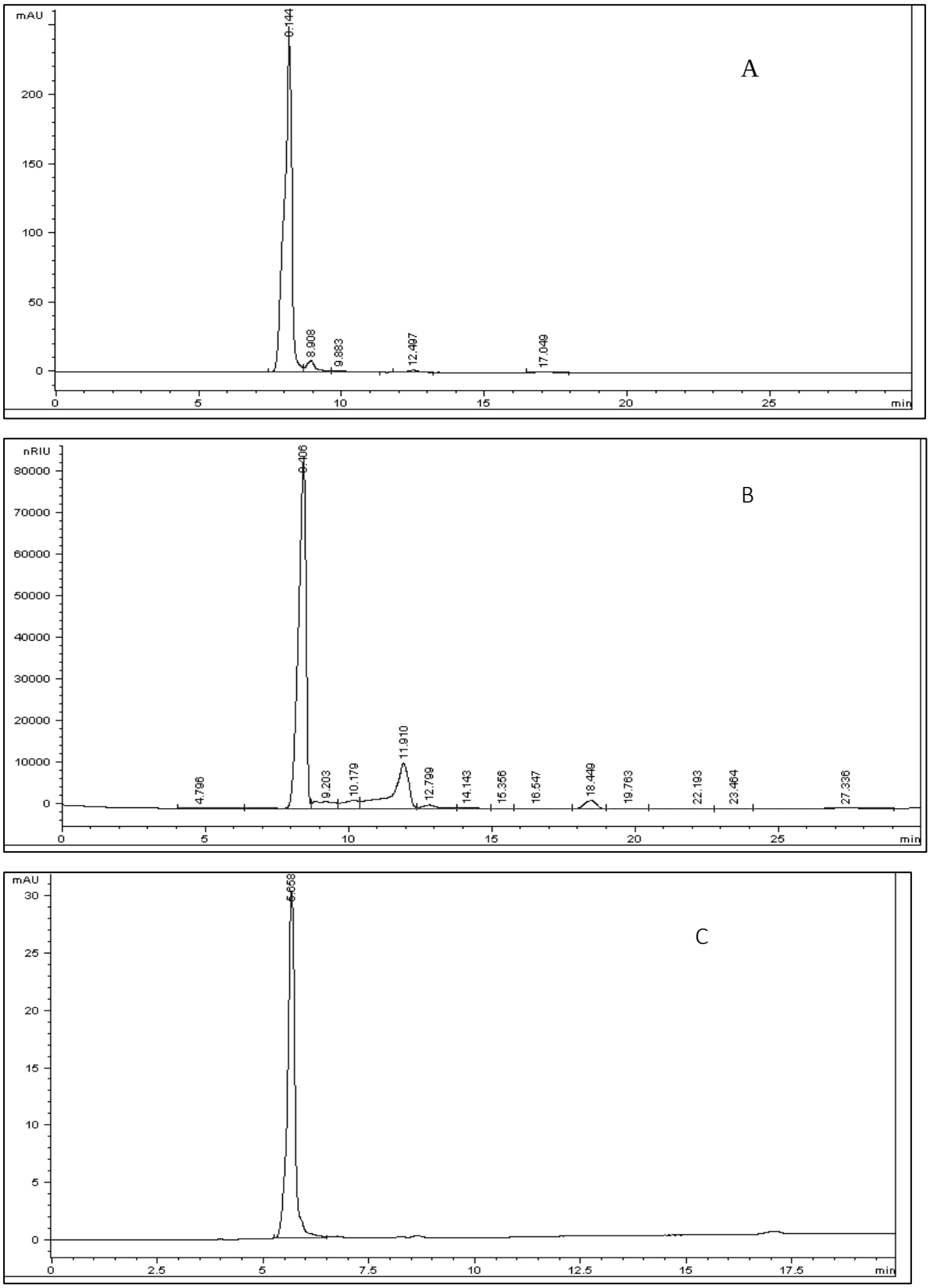

Figure 4. HPLC chromatogram of organic acids (A), sugars (B) and pectin (C) of the modified pectin dried with spray dryer.

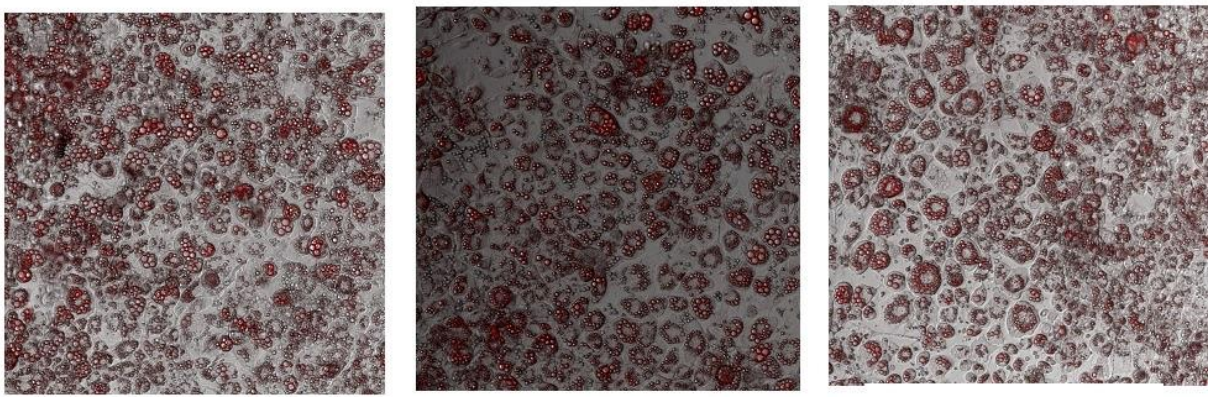

Figure 5. Samples of chitosan (left), pectin(center) and modified pectin(right) are demonstrated significant induction of cell differentiation during in vitro experiments.

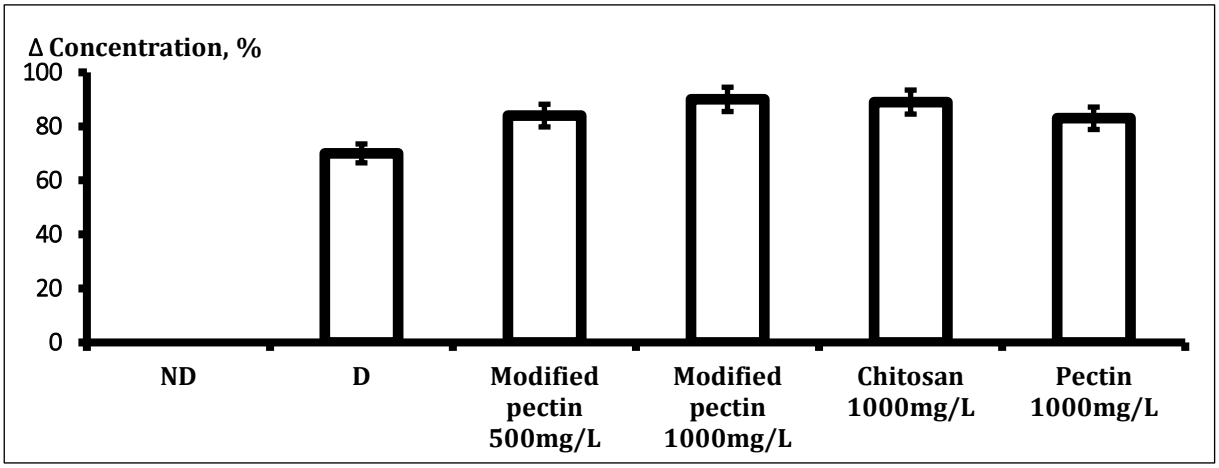

Figure 6. The adipogenesis in the 3T3-L1 cells was analyzed by AdipoRed staining treated with 1000 and 500 μg/ml of the modified pectin which showed pro-adipogenic dose-dependent activity.

Conclusions

This paper successfully has demonstrated that the films of starch-chitosan mixture do not have a toxic effect. There is growing interest in using other polysaccharides, such as pectin, for the medical application.

Generally, the MCP has a molecular weight of 10-20 KDa, a degree of polymerization of 30-70 units and a degree of esterification of less than 50%. In this way, modified pectins of high antitumor power are obtained. This study represents an initial assessment of the in vivo performance of hydrogels with pectin. Further studies are required to better elucidate the in vivo behavior of pectin.

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